Aspects on the ecology and dynamics of juvenile green turtles (*Chelonia mydas*) at foraging grounds of Culebra Archipelago, Puerto Rico
Progress Report for FY 02-03

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Introduction:

For the past three years, the Proyecto Carey-Estudio Peje-blanco have been conducting in-water surveys to evaluate the current status of the aggregations of green turtles (*Chelonia mydas*) at several feeding grounds in the Culebra Archipelago, Puerto Rico. The information gathered up to date, together with the data obtained during similar surveys on 1987-1989 (Collazo *et al.*, 1992) and 1998-1999 (José Rivera pers.com) would be useful for determining population and ecological parameters necessary for the implementation of the recovery plan for this species. Specially, data on size class composition, growth, and trends on population size would provide baseline habitat-species status variables from which subsequent threats to habitat and species integrity can be determined.

The following report summarizes the result for the fiscal year 2002-2003 together with comparisons of other seasons, when appropriate.

Methods and Results

Study site:

The Culebra Archipelago is located at 30 km from Puerto Rico's east coast (Fig 1). Sea grasses and coral reefs surround the Archipelago, which is composed of more than 9 cays. Three study sites were selected from previous studies. These sites are Mosquito, Puerto Manglar, and Culebrita (Fig 1). The depth of all sites varies from 8 to 15 meters.

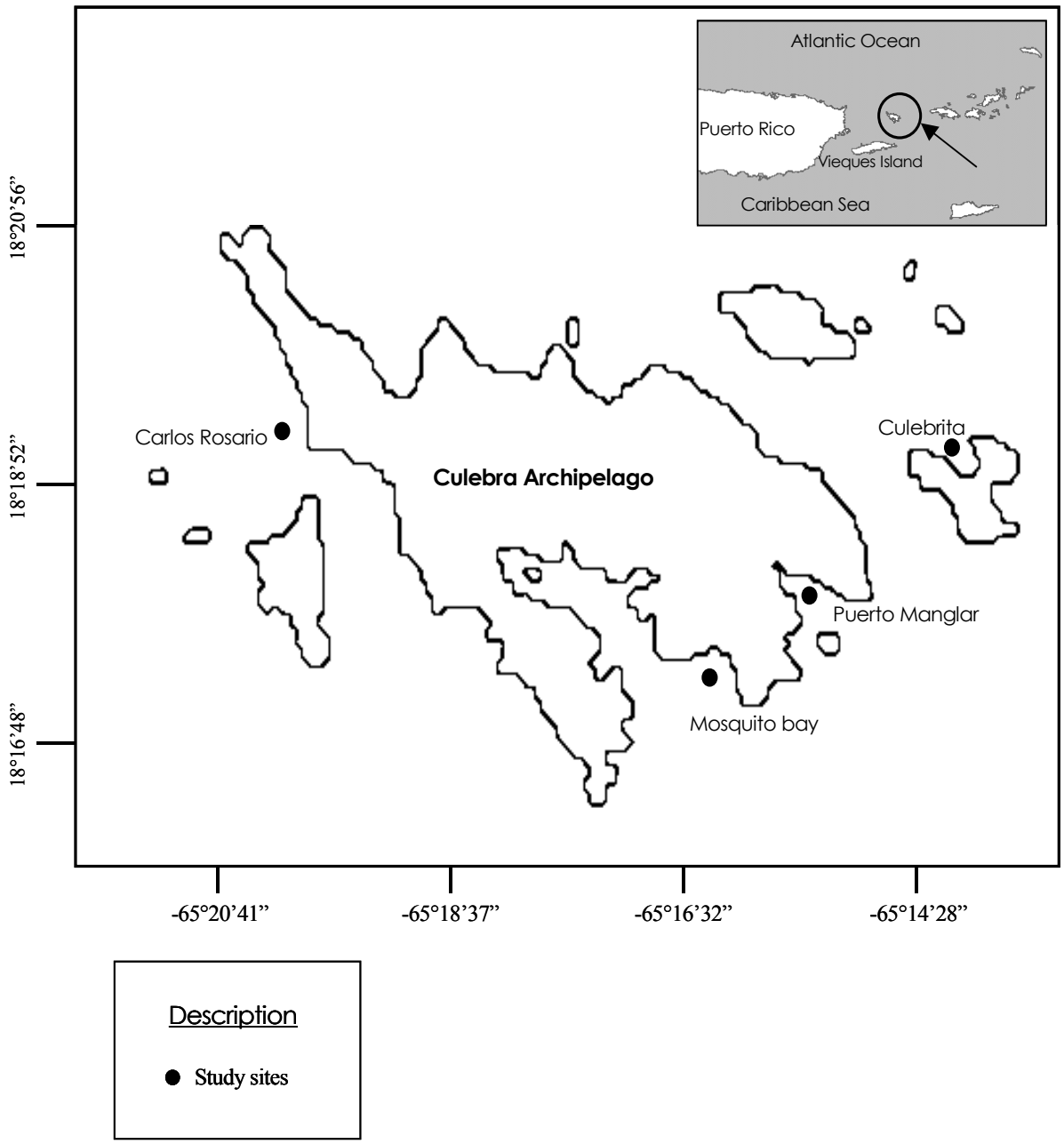

Figure 1. Map of the Culebra Archipelago, Puerto Rico. Black dots indicates the study sites of the project.

Turtle captures and morphometry:

We used two boats of approximately five meters length to conduct the surveys. The methodology for turtle captures was adapted from Collazo *et al.* (1992). At the study sites, a net of 200 m length and five meter depth (#18 nylon, 5m stretch mesh) was deployed parallel to the shore in an area with similar net-depth or shallower. An average of six swimmers snorkeled along the net to encounter the turtles. The noise produced by a boat engine, while carefully circuiting the shore, make turtles move toward the net. On Culebrita, three swimmers snorkeled from and to the shore looking for turtles, also assisted by chasing turtles toward the net. Once captured, each turtle was taken to an anchored boat for morphometry and tags protocols.

The net was set once at Pto Manglar (only in one ocassion it was set twice); and two times at Culebrita and Mosquito on each daily visit (session). During FY 2002-2003, two field trips were conducted (March and July 2003). The catch per unit of effort (CPUE) assessments was made by dividing the total number of turtle captures with the survey session (net set x day x area). Pto. Manglar has the highest CPUE, while no captured was obtained in Mosquito (Table 1). In order to evaluate trends of data collected in past years (Collazo *et al.* 1992); we calculated the effort (defined as catch per net set) by dividing the total number of turtle captures with the total sampling occasions during the given field season (Table 2). During FY 02-03 (2003), increases were observed for Manglar and Culebrita (Table 2) but Mosquito site, continue to be a concern, since the numbers of animals captured and/or sighted have decreased dramatically (Table 2) for the past 7 years. A comparison of habitat quality among used, randomly selected and unused sites should be performed in order to determine which character are limiting the use of sites by turtles.

Table 1. Green turtles captured per unit effort at Culebra, Puerto Rico, FY 02-03.

Site	Mean CPUE	STD	SE	N
Mosquito	0.00	0.00	0.00	0
Pto. Manglar	4.0	3.03	1.24	24
Culebrita	3	1.27	0.57	30

Table 2. Catch per net set of green turtles in-water surveys at Culebra Archipelago on each sampling period.

Site	Catch per net set on each sampling period			
	2003	2002	2001	1987-1989
Mosquito	0	0.25	0.38	2.16
Pto. Manglar	4.0	3.20	3.58	0.86
Culebrita	3.0	2.88	1.72	2.04

All turtles caught were measured with calipers to obtain straight lengths of the carapace, weighted and tagged on both flippers with small Monel and plastic tags (only turtles > 35 cm SCL) prior to release. In addition, turtles with no plastic tags were injected with Passive Integrated Transponder (PIT) tags; AVID brand.

During FY 02-03 field season a total of 54 green turtles were captured during the net sessions with 28 (52%) turtles being captured for the first time. Twenty-six turtles (48%) were recaptures from previous years (Appendix I). We expect to obtain greater recapture rates during the following surveys. This will allow us to calculate approximately the number of turtles using these sites among seasons. Recaptured turtles were obtained only within the same sites. This suggests that this turtles have great fidelity to these grounds, possibly acting as local populations with few or no displacement among sites. If this proof to be true, these grounds might be irreplaceable and protection from degradation should be mandatory.

No turtles were captured more than once in the same season but recaptures among seasons were obtained. This suggests that local populations can be accounted for a specific season by netting for shorter intervals (perhaps every other week during a two

month period). Also suggest that turtle may behave net-shy immediately after released but will eventually return to the sites.

Green turtles captured ranged in size from 40.7 to 83.7 cm SCL n-t (mean = 54.01 cm, SD =10.76). See Figure 2. Size distribution of the total of captures since 2000 (n=177) are shown in figure 3. All turtles were considered to be either juveniles or sub-adults due to their size and testosterone levels. These size classes appeared to be the only one using these grounds, evidenced by the one-modal shape graph of size distribution with smallest and largest size classes unrepresented. These characteristics suggest that the turtles are using this feeding ground as a developmental habitat.

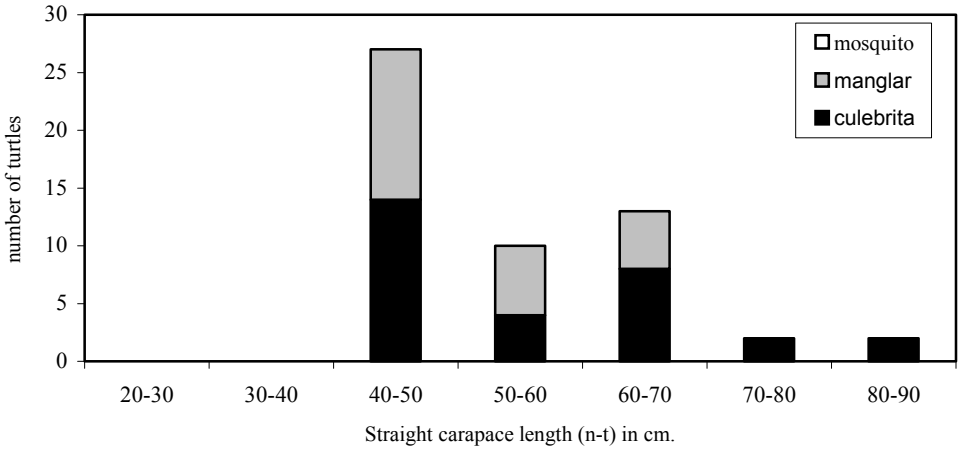

Figure 2. Size distribution of 54 green turtles captured at Culebra Archipelago: FY 02-03.

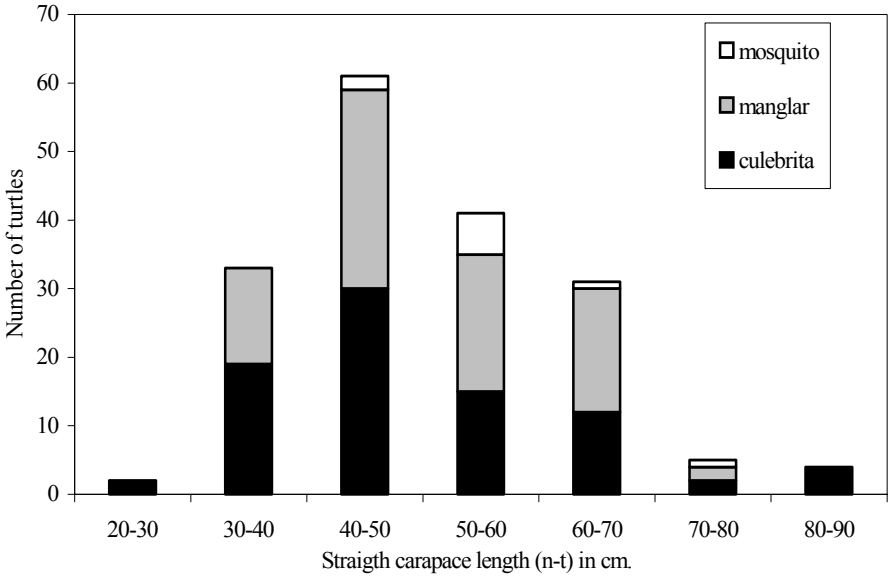

Figure 3. Size distribution of 177 green turtles at study sites, 2000-2003.

Long distance tag recoveries:

Last year we received reports of three long distance tag recoveries for green turtles tagged at Culebra Archipelago. Two turtles were reported by Cynthia Lagueux (Caribbean Conservation Corporation). These turtles were caught by fishermen at Miskito Cays, Nicaragua. The exact locations were as follows: one of the turtles (tag# PPW017) was captured on October 9, 2002 at Sandy Bay Sirpi, which is located north of the mouth of the Rio Grande de Magalpa. The other turtle (PPW059) was caught on October 2001 at Tasba Pauni, which is located south of the mouth of Rio Grande de Matagalpa (near the mouth of Pearl Lagoon). A third turtle was reported by Claudia Ceballos (INVEMAR, Colombia) and fishermen on Bahía Honda, Peninsula de la Guajira, north of Colombia, captured the turtle. Unfortunately, for all three cases there was no more information on size of the turtles. However, all three turtles were classified as juvenile/sub adults when first captured (size range: 39.6-54.3 cm SCL). By

extrapolation we concluded that they were in the adult-size class when captured (see next section). Tags were in good condition. Table 3 summarizes the capture records.

Table 3. Dates and locations of tag recoveries

Tag Number	First captured date and location	Last captured date and location
PPW017	2 Aug 1990-Culebrita, PR	9 Oct 02-Miskito, Nicaragua
PPW059	19 June 1991-Manglar,PR	Oct-01-Miskito, Nicaragua
AAL161	13 Feb 1987-Mosquito,PR	Jan-03-Guajira-Colombia

Growth rates:

The study of growth rates is essential for estimating the time it takes an animal to reach maturity, and therefore to start reproducing. Growth information allows us to make predictive population models that can help us make better management decisions. Growth data obtained from Collazo *et al.* (1992) reported a different pattern than those reported for the Atlantic green turtles. The tagging effort of this project is providing a baseline for growth rates data and would contribute to determine if the discrepancy between the reported growth rates is valid or simply the result of a limited size sample. We measured the straight carapace length (SCL, n-t) in cm of all turtles captured using two Haglof tm 65 and 95 cm tree calipers. To minimize measurement errors, the same observers (AOR or CED) took all measurements. Recaptured turtles were identified by their external tags or PIT tags detection.

A total of 42 turtles were recaptured in our study area since 2001. The time interval between captures ranged from 258 to 2150 days. The growth rates calculated for captures over an interval greater than 8 months are presented in Figure 4. These rates were variable, ranging from 1.01 to 11.09 cm/year (mean = 6.16, SD 1.83). The result in this study indicates a much faster growth rates compare with other areas in the Atlantic Ocean (Bjorndal & Bolten., 1988). We are currently in the process of gathering more data on growth rates to establish relationships with sex, habitat effect, and turtle density.

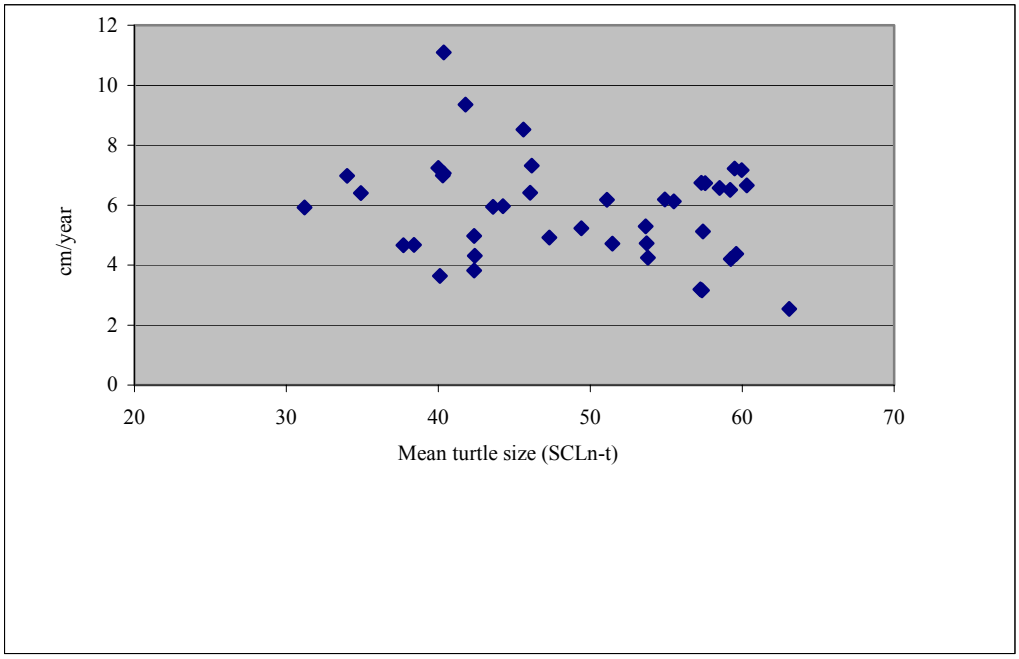

Figure 4. Growth rate based on 42 recaptures at all study sites in Culebra Archipelago.

Sex ratios:

The sex of individual immature green turtles captured was obtained by measuring blood serum testosterone levels and comparing the values with other studies in the West Atlantic Ocean, such as Bermudas and Bahamas (Meylan, unpublished data; Bolten *et al*., 1992), which were determined by laparoscopic observation of the gonads. According to those studies the maximum testosterone level indicative of a female was 100 pg/ml, while 200 pg/ml was the minimum for a male (Owens, per. com). Intermediate results between these two values were catalogued as inter-sex. Blood samples were taken from the cervical sinus (Owens and Ruiz, 1980) employing Vacutainer sterile vacuumed tubes and placed in an ice cooler. After the serum was separated, it was collected with a pipette and placed in sterile 1 ml vials to be frozen until analyzed. All of our samples were processed using radioimmunoassay techniques (Owens *et al*. 1978) by the staff of David Owens of the Grice Marine Lab at College of Charleston, South Carolina-USA.

Serum samples from 52 individual turtles collected during the past two seasons were analyzed for testosterone levels to determine sex (see Appendix IV). The percentage of female was 65.3%, the percentage of males was 23.1% and indeterminate individuals constituted 11.6%. If we excluded the indeterminate individuals, the sex ratio was skewed towards females (2.8:1).

Fibropappillomas:

Marine turtle fibropapillomatosis (FP) is one of the most important disease affecting wild marine turtles (Aguirre *et al*. 2002). Since 2000, we have been reporting turtles with tumors at Puerto Manglar. Almost all the tumors observed have been pathologically confirm as FP (Report 2002). The amounts of turtles in this study site with FP or look-alike tumors have been increasing. We reported 3 cases in 2001, 8 cases in 2002 and 16 during this season (see appendix III). Collaborative work is currently conducted with several researchers. George Balazs (NMFS-Hawaii) and Thierry Work (U.S. Geological Survey) are aiding in the identification of tumors; James Casey (Univ. of Cornell) is also analyzing biopsies of the tumors for virology and other pathological examinations; and finally, Fernando Torres from Univ. of Georgia is validating non-invasive techniques for collecting samples from the turtles and comparing an aggregation of green turtles with no known prevalence of gross FP lesions (Culebrita) with another aggregation of green turtles with high prevalence of conspicuous lesions (Pto. Manglar); see Appendix V.

It is important to note that even though the pathology of FP varies with the presence or absence of environmental factors, published observations agreed in that FP is highly prevalent in habitats that are proximal to agricultural and urban development (Balazs and Pooley, 1991; Herbst, 1994; Limpus and Miller, 1994). That might be the case in Pto. Manglar; a close, shallow non-dynamic bay partially enclosed by an urban development area (formerly an agricultural zone). This is the only green turtle developmental ground in Culebra where FP is reported. Although Pto. Manglar is in close proximity to Culebrita, no FP was detected in the latter. This again suggests high fidelity of individual

turtles to their developmental grounds and that FP (found in nearly 70% of the green turtles of Manglar; see appendix III) might not migrate freely as "water-borne" viruses or otherwise, a greater detection of the tumors was expected outside Manglar. This virus is probably transmitted directly (contact) or indirectly (open seizures near the vector, feces, etc.) from turtle to turtle. It is known that many species of herpesvirus can be transmitted by contact of connective tissue (ex, around eyes, mouth, and cloacae) or open wounds (or abrasions) with infested feces and/or body fluids. A study to determine the presence of herpesvirus on turtle feces, is highly recommended.

Hawksbill surveys:

In addition to the surveys to assess green turtle aggregations, hawksbill in-water surveys were conducted in Carlos Rosario Marine Reserve at north east of mainland Culebra. Since captured methods and habitat characteristics are different, we keep both studies separated (green turtle study and hawksbill study). The following information summarized our surveys for hawksbill turtle during the 2003 field season with data from other years when relevant. Turtle surveys consisted of two or three hour long, daytime snorkeling censuses in hard-bottom and coral reef habitats with a depth of 15 m or less (see Figure 1 of site location). Turtles were captured by hand following the method of Diez and van Dam (1994) by three to 7 swimmers, followed by one person on board a boat. All captured turtles were brought to the vessel for data collection. Turtles sighted (but not captured) were also recorded for each survey. The capture per unit effort was calculated by dividing the number of sighted turtles (whether captured or not) by the total time of each survey (hours). Maximum straight carapace length (SCL) in cm was obtained from all turtles using a Haglof 60 cm tree caliper. To minimize measurement errors, the same observer took all measurement and another wrote all the notes.

Turtles greater than 25 cm (SCL) were tagged in both front flippers using Inconel tags and turtles greater than 35 cm (SCL) were tagged in one of the front flippers with plastic tags (Dalton JUMBO ROTO tags). Passive Integrated Transponder tags were inserted to animals less than 25 cm or turtles with only Inconel tags. All turtle captures location

were recorded with a Global Position System receiver and later released to the same location originally caught.

During the 2003 field season, a total of 20 hawksbills were caught during two survey sessions of 2 and 3 hours each for a CPUE of 3.94 turtles per hour (see appendix II). Table 4 indicates the CPUE for hawksbill turtles by year. The size range for turtles captured during FY 02-03 ranged from 23.7 to 39.0 cm SCL. Three were recaptures from other years. Based on this data, we concluded that Carlos Rosario Marine Reserve is an important recruit and developmental habitat for hawksbill turtles in Puerto Rico.

Table 4. CPUE for hawksbill turtles captured at Carlos Rosario Marine Reserve, Culebra.

Year	Hours of survey	Number of hawksbill (captured and sighted)	CPUE
1997	1.5	3	2.00
2000	7.5	10	1.33
2001	2.0	7	3.50
2002	6.6	4	2.00
2003	5	20	5
Total	19.6	44	2.24

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Appendix I. List of green turtles captured during FY 02-03 at Culebra Archipelago.

date	site	left tag	rigth tag	Pit tag .	New?	SCLn-t (cm)
3-Mar-03	culebrita I	ppy335	ppm416	114409270A	R	66.8
3-Mar-03	culebrita I	bp9167	xxp781	040076265	R	63.2
3-Mar-03	culebrita I	bp9168	xxp782	050017082	R	46.1
3-Mar-03	culebrita I	bp9166	xxp780		N	41
3-Mar-03	culebrita I	bp9165	xxp778		N	55.3
3-Mar-03	culebrita I	bp9155	xxp730		R	84.2
3-Mar-03	culebrita I	xxp779	bp9127		R	44
3-Mar-03	culebrita I	bp9164	xxp777		N	63.8
3-Mar-03	culebrita I	bp9163	xxp776		N	48.3
3-Mar-03	manglar	ppy326	ppm424	114516155A	R	66.1
3-Mar-03	manglar	bp9157	xxp774		N	51
4-Mar-03	culebrita I	bp9173	xxp787		N	53
4-Mar-03	culebrita I	bp9172	xxp786		N	84
4-Mar-03	culebrita I	bp9141	xxp758	050085321	R	59.3
4-Mar-03	culebrita I	bp9143	xxp759	050122345	R	46.2
4-Mar-03	culebrita I	bp9171	xxp785	050307346	R	40.3
4-Mar-03	culebrita I	bp9170	xxp784		N	44.3
4-Mar-03	culebrita I	bp9158	xxp788		N	70.9
4-Mar-03	culebrita I	bp9169	xxp783		N	44.8
4-Mar-03	manglar	bp9159	xxp789		N	40.5
4-Mar-03	manglar	bp9176	xxp792		N	49.6
4-Mar-03	manglar	bp9175	xxp791		N	43.5
4-Mar-03	manglar	bp9174	xxp790		N	46.4
4-Mar-03	manglar	xxp773	bp9129		R	47.9
4-Mar-03	manglar	bp9152	xxp764	050289784	R	65.9
5-Mar-03	culebrita I	bp9177	xxp793		N	62.9
5-Mar-03	culebrita I	bp9178	xxp794		N	69.8
5-Mar-03	culebrita I	bp9134	xxp708	050315894	R	45.6
5-Mar-03	culebrita I	xxn879	xxn878	050273342	R	63.3
5-Mar-03	culebrita I	bp9179	xxn897	113727217A	R	66.1
5-Mar-03	culebrita I	bp9162	xxp766	050265800	R	44
5-Mar-03	manglar	bp9180	xxp795		N	43.9
5-Mar-03	manglar	bp9184	xxp798		N	60.4
5-Mar-03	manglar	bp9183	xxp797		N	58.4
5-Mar-03	manglar	bp9181	xxp796		N	44
5-Mar-03	manglar	xxn876	bp9182	050302573	R	52.8
5-Mar-03	manglar	ppm221	ppm227	040103091	R	69.2
8-Jul-03	culebrita I	bp9195	rra223		N	66.9
8-Jul-03	culebrita I	bp9134	xxp708	050315894	R	47.3
8-Jul-03	culebrita I	rra226	bp9127		R	45.2
8-Jul-03	manglar			050071527	R	50.4
8-Jul-03	manglar	bp9199	rra228		N	53.9
8-Jul-03	manglar	bp9198	rra227		N	47.2

Appenix I (continuation)

Date	Study site	Tag (L)	Tag (R)	PIT tag	New?	SCL cm
8-Jul-03	manglar	rra225	bp9129		N	49.7
8-Jul-03	manglar	bp9196	rra224		N	61.2
8-Jul-03	manglar	bp9181	xxp796		R	46.2
8-Jul-03	manglar	bp9159	xxp789		R	42.8
8-Jul-03	manglar	bp9197	rra275		N	50.8
9-Jul-03	culebrita I	bp9144	bp9133	050319332	R	43.2
9-Jul-03	culebrita I	bp9200	rra229		N	43.8
9-Jul-03	culebrita I	bx1401	rra230		N	59.4
9-Jul-03	culebrita I	ppy338	ppm244	113726677A	R	66.3
9-Jul-03	manglar	rra231	bp9136		R	48.7
10-Jul-03	manglar	ppm267	nnw247		R	48.3

Appendix II. List of hawksbills captured at Carlos Rosario, Culebra: FY 02-03

date	Site	left tag	right tag	Pit tag .	New?	SCLn-t (cm)
6-Mar-03	carlos rosario	rra225	xxp800	050008274	New	28.2
6-Mar-03	carlos rosario	xxp772	bp9156		Recapture	38.5
6-Mar-03	carlos rosario	rra260	rra254	050260115	New	32.7
6-Mar-03	carlos rosario	rra256	xxp799	050279768	New	25.7
6-Mar-03	carlos rosario	rra258	rra251	050053625	New	31.7
6-Mar-03	carlos rosario	rra257	rra252	050116335	New	26.8
6-Mar-03	carlos rosario	xxp751	xxp752		Recapture	35.8
6-Mar-03	carlos rosario	rra259	rra253	050079559	New	23.8
7-Mar-03	carlos rosario	bp9186	raa261		New	38.6
7-Mar-03	carlos rosario	xxp771	xxp770	040103381	Recapture	35
7-Mar-03	carlos rosario	xxp750	bp9140		Recapture	39.7
7-Mar-03	carlos rosario	bp9185	rra262		New	32.2
7-Mar-03	Luis Penha	bp9187	rra263		New	39.9
7-Mar-03	Luis Penha	rra264	rra265	050084826	New	32.8
7-Mar-03	Luis Penha	rra267	rra266	050037883	New	31.6
7-Mar-03	Luis Penha	bp9188	rra268		New	45.3
10-Jul-03	carlos rosario	ppm204	ppm203	055817565	Recapture	38.4
10-Jul-03	carlos rosario	rra258	rra251	050053625	Recapture	32.8
10-Jul-03	carlos rosario	rra232	rra233	055809586	New	31.5
10-Jul-03	carlos rosario	xxp771	xxp770	040103381	Recapture	36.3

Appendix III. List of green turtles with FP captured at Culebra Archipelago during FY 02-03.

date	Site	New?	left tag	right tag	Pit tag #	SCLn-t (cm)
8-Jul-03	manglar	Recapture			050071527	50.4
10-Jul-03	manglar	Recapture	ppm267	nnw247		48.3
3-Mar-03	manglar	Recapture	ppy326	ppm424	114516155A	66.1
9-Jul-03	manglar	Recapture	rra231	bp9136		48.7
4-Mar-03	manglar	New	bp9174	xxp790		46.4
4-Mar-03	manglar	Recapture	xxp773	bp9129		47.9
8-Jul-03	manglar	New	rra225	bp9129		49.7
3-Mar-03	manglar	New	bp9157	xxp774		51
8-Jul-03	manglar	Recapture	bp9181	xxp796		46.2
5-Mar-03	manglar	New	bp9184	xxp798		60.4
5-Mar-03	manglar	New	bp9181	xxp796		44
8-Jul-03	manglar	New	bp9199	rra228		53.9
5-Mar-03	manglar	Recapture	xxn876	bp9182	050302573	52.8
8-Jul-03	manglar	New	bp9196	rra224		61.2
8-Jul-03	manglar	New	bp9197	rra275		50.8
4-Mar-03	manglar	Recapture	bp9152	xxp764	050289784	65.9

APPENDIX IV. List of green turtles with testosterone level and predicted sex at Culebra, PR:2001-2003.

Turtle Tag ID	T (pg/ml)	Predicted sex	Turtle Tag ID	T (pg/ml)	Predicted sex
142	5.7	F	PPM265	6.57	F
291	5.03	F	PPM408	0.223	F
386	6.02	F	PPM429	9.34	M
609	4.57	F	PPY328	6.28	F
885	3.93	F	PPY380	10.18	M
7271	14.65	M	XX 761,760	5.36	F
9141	6.64	F	XX 868,869	9.53	M
9147	9.97	M	XXN802	0.1	F
9148	11.86	M	XXN803	10.75	M
9153	5.88	F	XXN809	7.04	F
9155	7.03	F	XXN811	6.09	F
9160	6.18	F	XXN814	7.53	U
9162	6.65	F	XXN815	8.63	M
BP9133	6.93	F	XXN817	5.23	U
BP9143	3.5	F	XXN825	8.99	U
BP9152	6.86	F	XXN826	10.97	M
BP9154	9.22	U	XXN856	7.03	F
NNW244	6.59	F	XXN858,859	6.5	F
PPM206	8.91	M	XXN860	3.31	F
PPM209	5.33	F	XXN863	4.61	F
PPM213	8.68	M	BP9450	5.99	F
PPM214	1.16	F			
PPM218	6.87	U			
PPM220	5.82	F			
PPM231	10.5	M			
PPM233	5.24	F			
PPM236	6.54	F			
PPM241	6.53	U			
PPM245	0	F			
PPM250	7.03	F			
PPM262	5.57	F			

Appendix V. Report on fibropapillomosis in marine turtles at Culebra Archipelago, PR

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Abstract

Fibropapillomatosis (FP) is a debilitating proliferative disease caused by a herpesvirus, which in recent decades has become a growing threat to the health of endangered sea turtle populations around the world. This study used nested PCR and primers directed at a fragment of the viral DNA-polymerase gene to detect the presence of Green Turtle Herpesvirus (GTHV) in skin and tumor tissues of two species of endangered sea turtles, green sea turtles (*Chelonia mydas*) and hawksbills (*Eretmochelys imbricata*), in Puerto Rico. This technique was applied to determine the presence of GTHV in a population of green turtles with no known prevalence of gross FP lesions as compared to a population of green turtles with high prevalence of gross lesions. Also, a skin swab technique for the detection of FP was evaluated for its usefulness in detecting GTHV.

Introduction

Fibropapillomatosis in sea turtles, also called Green Turtle Fibropapillomatosis (GTFP) because of its high prevalence in endangered green sea turtles, is a disease characterized by the proliferation of benign but cutaneous fibropapillomas and occasional internal fibromas[1]. The fibropapillomas, which are frequently located on the head, neck, and flippers, can vary in size and can severely limit the turtle's ability to move and find food[2]. Additionally, the tumors may place affected turtles at greater risk to other dangers such as predation, entanglement in debris, and stranding[2]. The juvenile size-class seems to be the most seriously affected population group[3]. In the Caribbean, almost a decade ago, 30% of stranded or captured green sea turtles were reported to have skin tumors, and in recent years, the occurrence of fibropapillomatosis has been rapidly increasing around the world[4].

Current evidence strongly supports an alpha-herpes virus (GTHV) as the etiologic agent in FP. Herpesvirus sequences have been successfully detected in greater than 90% of tumor tissues of affected turtles by polymerase chain reaction (PCR)[5]. In addition, fibropapillomas are able to be induced by

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[1] Lackovich JK, Brown DR, Homer BL, Garber RL, Mader DR, Moretti RH, Patterson AD, Herbst LH, Oros J, JacobsonER, Curry SS, Klein PA. (1999) Association of herpesvirus with fibropapillomatosis of the green turtle *Chelonia mydas* and the loggerhead turtle *Caretta caretta* in Florida. Dis Aquat Organ 30; 37(2): 89-97.

[2] Herbst LH. (1999) Marine turtle fibropapillomatosis: Hope floats in a sea of ignorance. Proceedings of the 19th Annual Sea Turtle Symposium, South Padre Island TX, 39-40.

[3] Murakawa SKK, Balazs GH, Ellis DM, Hau S, and Eames SM. (1999) Trends in Fibropapillomatosis among green turtles stranded in the Hawaiian Islands, 1982-98. 19th Annual Sea Turtle Symposium, 239-241.

[4] Williams Jr. EH, Bruckley-Williams L, Peters EC, Pinto-Rodriguez B, Matos-Morales R, Mignucci-Gianonni A, Hall KV, Rueda-Almonacid JV, Sybesma j, Bonnelly de Calventis I, Boulon RH. (1994) An epizootic of cutaneous fibropapillomas in green turtles *Chelonia mydas* of the Caribbean: Part of a Panzootic? J Aquatic Animal Health 6: 70-78.

[5] Quackenbush SL, Casey RN, Murcek RJ, Paul TA, Work TM, Rovnak J, Limpus CJ, Chaves A, DuToit L, Aguirre A, Spraker TR, Peres JV, Vermeer LA, Horrocks JA, Balazs GH, Casey JW. (2000) Quantitative Fluorogenic Real-Time PCR Assessment of Herpesvirus Sequences from Normal Tissue and Fibropapillomas of Turtles Sampled at Different Geographic Locations. Proceedings of the 20th Annual Sea Turtle Symposium, Orlando FL: 2000: 194-195.

intradermal inoculation with a cell-free tumor tissue homogenate, and herpesviral particles have been observed in experimentally induced tumors using electron microscopy[6,5]. Nevertheless, virus has not yet been isolated in cell cultures, and the pathogenesis, epidemiology, and most of the disease biology remains obscured.

The goal of this project is to document the prevalence of FP as well as the herpesvirus that causes FP, in green and hawksbill turtles at three sites in and around the island of Culebra, Puerto Rico. Two of these study sites, Puerto Manglar and the island of Culebrita, are situated in close geographic proximity of each other but demonstrate significant differences in prevalence of FP. Since the course of this disease is prolonged and appears to involve periods of dormancy and tumor regression, infected animals may not always show evident cutaneous proliferation. One objective of this project was to determine the presence of GTHV in the grossly unaffected green turtle population. The other objective is to test a skin swab protocol for its usefulness in detecting GTHV in the skin of affected and unaffected sea turtles[7].

It is hoped that the information obtained from this study will help to address the current lack of FP prevalence studies in these areas of Puerto Rico, which are known to provide important habitat for endangered sea turtles, and eventually aid in the management of these populations.

Materials and Methods

A survey of fibropapillomatosis in Green and Hawksbill sea turtles was conducted in cooperation with the Departamento de Recursos Naturales y Ambientales of Puerto Rico (DRNA-PR) during June and July of 2003. Skin biopsies and skin swabs were collected from the turtles sampled during a routine semi-annual survey conducted by the DRNA-PR endangered species program.

These surveys took place at study sites on the islands of Culebra and Culebrita. The island of Culebra is located 27 km east of the main island of Puerto Rico and measures approximately 11 km in length by 5 km in width, with an approximate area of 26 km^2. It is closely surrounded by 23 smaller islands, the largest of which is the island of Culebrita.

Study sites consisted of Puerto Manglar, Carlos Rosario, and the island of Culebrita.

Green turtles were collected from Puerto Manglar and the small island of Culebrita. All hawksbill turtles (*Eretmochelys imbricata*) were collected in coral reef habitat at Carlos Rosario, Culebra.

Twenty-four live turtles were sampled in this study – seventeen green sea turtles (*Chelonia mydas*), seven hawksbills (*Eretmochelys imbricata*). Seven of the green turtles were collected from Culebrita, only one of which demonstrated a cutaneous papillomatous or verrucous mass. The turtle collected from Culebrita (tag # PPM244, PPM338) was observed to have only two small, smooth round masses of less than 0.5 cm located on the lower eyelid of the left eye. Biopsies were not taken from these masses due to their close proximity to the eye. In addition, the masses observed on one of the seven affected turtles collected at Puerto Manglar were located solely on the skin in close proximity to the eye, and no biopsy was taken from this mass either.

The remaining ten were collected from Mosquito Bay, Puerto Manglar, of which seven showed cutaneous tumors resembling fibropapillomas. As a negative control, skin samples were taken from one dead juvenile loggerhead (*Caretta caretta*) that was submitted to the University of Georgia for post-mortem.

Collection of Samples

All surveys for this project were conducted by trained biologists employed by the DRNA-PR and US Fish and Wildlife Service, who followed established protocols in accordance with federal regulations.

To determine the presence of FP in the green sea turtle populations of Puerto Manglar and the island of Culebrita, surveys were conducted on July 8th, 9th, and 10th, 2003. At these study sites, a net of 200 meters length x 5 meters depth (#18 nylon, 16" stretch mesh) was deployed parallel to the shore in an area

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[6] Lu Y, Aguirre AA, Work TM, Balazs GH, Nerurkar VR, and Yanagihara R. (2000) Identification of a small, naked virus in tumor-like aggregates in cell lines derived from a green turtle, *Chelonia mydas*, with fibropapillomas. Journal of Virological Methods 86: 25-33.

[7] Maggi RG, Yoarrys R, Cordero J, Deya S, and Diez, CE. A quick, low cost, and harmless sampling method for the detection of Herpesvirus infection in green sea turtles. Unpublished

with similar net depth, sometimes shallower. An average of 7 (range 6-10) surveyors snorkeled along the net watching for turtles. Once captured, each turtle was taken to one of the boats where morphometric and identification data was recorded and unidentified turtles were fitted with a flipper tag or subdermal PIT tag. During each session, the net was lowered once at Mosquito Bay, Puerto Manglar, and twice at Culebrita.

After careful gross inspection for tumors, one dermal biopsy was obtained from each turtle in the area of unaffected skin lateral to the neck (and medial to the front flipper), using a 6mm punch biopsy[8]. Prior to biopsy, the area was aseptically prepared using a betadine scrub and alcohol. The biopsy tissue was removed with an 18 gauge needle and scalpel blade and placed epidermis up on a 3x3 in. square of cotton gauze, on which it was then cut in half. One half of the biopsy was fixed in 10% formalin for histopathologic examination and the other was frozen for molecular analysis (PCR). If the turtle demonstrated tumors, an additional biopsy, this time from the tumor itself, was taken in exactly the same manner stated above and stored in the same way, and then the turtle was released. All turtles were released at the same site of capture.

Because the hawksbill species is rarely observed to be affected by FP, these turtles were sampled to serve as a "negative control population." Surveys were conducted at Carlos Rosario on June 10th and 20th, and July 10th, 2003. Snorkelers patrolled coral reef habitat in search of turtles, swimming parallel to the coastline for one to two hour periods. Hawksbills were captured by hand, and once captured, each turtle was taken to one of the boats where morphometric and identification data was recorded and unidentified turtles were fitted with a flipper tag or subdermal PIT tag. One dermal biopsy was obtained from each turtle in the exact manner described above for green turtles, and the turtle was released. All turtles were released at the same site of capture.

To validate the diagnostic protocol proposed by Maggi, skin swabs were taken from the neck, axillary, and inguinal areas of each animal biopsied. The swabs were obtained by gently scrubbing approximately 4 cm^2 of epidermis with a PBS (phosphate buffered saline) soaked cotton swab. After sampling, each swab tip was broken off into a pre-labeled 1.5mL eppendorf tube containing 500µl of PBS. If a turtle exhibited tumors, an additional skin swab was taken directly from the tumor itself in exactly the same manner.

Nitrile gloves were worn by all handlers during sampling, and gloves were changed between turtles. Samples were collected from each turtle in the following order: normal skin swab, normal skin biopsy, tumor swab, tumor biopsy.

Histopathology Examination

Formalin fixed biopsies were embedded in paraffin, sectioned (5µm), mounted in charged glass slides and stained with hematoxylin and eosin. Slides were then examined under the microscope by a trained pathologist.

Molecular Analysis

To detect the presence of Green Turtle Herpesvirus (GTHV) in skin and tumor tissues, the DNA extracted from skin and tumor samples was subjected to a nested PCR assay using primers directed against a fragment of the herpesviral DNA-polymerase gene. Primer sequences used in the primary and nested PCR in this study are those previously described by Y. Lu, *et al*. 2000[9]. Presence of the GTHV DNA-polymerase gene fragment was evidenced by visualization of the predicted band, of 445-bp for primary PCR or 206-bp for nested PCR, after separation of PCR products by gel electrophoresis and staining with ethidium bromide. DNA extraction and PCR took place at the University of Georgia, College of Veterinary Medicine, Pathology Department in Athens, Georgia.

DNA extraction:

[8] Dutton PH, (1996) Proceedings of the International Symposium on Sea Turtle Conservation Genetics, US Department of Commerce, NOAA Tech Memo NMFS-SEFSC-396: p.19.

[9] Lu Y, Wang Y, Yu Q, Aguirre AA, Balazs GH, Nerurkar VR, Yanagihara R. (2000) Detection of herpesviral sequences in tissues of green turtles with fibropapilloma by polymerase chain reaction. Archives of Virology 145: 1885-1893.

DNA was isolated from the frozen biopsy tissues and swab samples using the commercially available, QIAGen® DNeasy Tissue Kit, according to manufacturer instructions. Biopsy tissues were cut into smaller pieces with a sterile surgical blade prior to digestion at 55°C for 24hrs. Approximately 20mg of tissue were used if biopsy size allowed, otherwise, all available tissue was included. The QIAGen® "protocol for animal tissues" was followed for the extraction of DNA from the frozen biopsy tissues. The final elution volume was 200µl, eluted 100µl at a time in two separate steps. The first 100µl elution step products were used in the subsequent PCR reactions.

DNA was extracted from swab samples following the QIAGen® "protocol for cultured animal cells." Prior to DNA extraction, swab samples were thawed to room temperature. Each swab was then agitated in 500µl PBS buffer, after which the swab was removed, and the tube was centrifuged at 6000rpm for 5 minutes to form a pellet. Agitation and pelleting steps were repeated once more before the PBS buffer was removed from the pelleted sample by careful pipetting. Following the protocol described by the manufacturer, 200µl PBS buffer was added to the pellet, along with digestion enzymes and buffer, and this mixture was incubated at 70ºC for 2 hours. The final elution volume of the extractions was 200µl, eluted separately in two steps, with 100ul eluted in each step. The first 100µl elution was used for the PCR reactions.

The DNA concentration of each extraction was measured using an Ultrospec 3000 UV/visible spectrophotometer manufactured by Pharmacia Biotech. An automated setting for detection of DNA was used.

Primary PCR amplification:

Detection of the fragment of the GTHV DNA-polymerase gene in the tumor and non-tumor swabs and biopsy tissues was performed using a nested PCR assay. For the detection of the primary GTHV sequence (445bp), 1µl of sample DNA extract was added to a 20ul reaction mixture composed of 1µl of each primer (5'-AGCATCATCCAGGCCCACAATCT-3' and 5'-CGGCCAGTTCCGGCGCGTCGACCA-3'), 10µl *Taq* PCR Master Mix (QIAGen®), and 7µl distilled water. A GeneAmp PCR System 2400 thermocycler, manufactured by Perkin Elmer®, was used for all PCR reactions, with reaction conditions as follows: 94ºC, 5 minutes initial denaturation, followed by 45 cycles of 1 minute denaturing at 94°C; 1 minute of annealing at 55°C, and 1.5 minutes of extension at 72°C. The PCR reaction was then completed by 7 minutes of extension time at 72ºC, and then held at 4°C.

Nested PCR reaction:

To perform nested PCR, 1 µl of the primary PCR amplification was used as a template, with a reaction mixture similar to the one described above except that the nested PCR reaction was performed using primer sequences 5' -CTGCTGACCGACTGGCTGGC-3' and 5'-AGCATGTCGCGCCCTACGGTGGTGAC-3'. The reaction conditions were the same as those used in the primary amplification described above. The nested PCR product was a 206bp fragment.

PCR products were size fractionated on 2% agarose gels by electrophoresis. The gels were stained with ethidium bromide, and viewed and photographed using an AlphaImager 2200 MultiImage light cabinet and software manufactured by Alphainnotech Corporation®.

Results

Biopsies

A total of 7 and 10 green sea turtles were sampled from Culebrita and Manglar respectively. Regardless of the site of collection, skin biopsies from grossly normal areas of skin in all green sea turtles were negative for GTHV by primary and nested PCR. Of all green turtles sampled, all (7) individuals from the Culebrita population, and 3 out of 10 from the Puerto Manglar population were negative grossly and by PCR. Similarly, all hawksbills sampled (7) had neither gross evidence of FP nor PCR evidence of GTHV. Seventy percent (7/10) of individuals sampled from the Puerto Manglar population had gross lesions consistent with FP. PCR results from these lesions are depicted in table 1.

Table 1. PCR of tumors from green turtles sampled at Puerto Manglar, a population known to be affected

Sample ID	Primary PCR result	Nested PCR result
G5	+	+
G7	+	+
G8	+	+
G10	-	-
G16	+	+
G17	-	-

Swabs

Standard and nested PCR results of swabs from unaffected skin in both populations of green turtles and in hawksbills, as well as from tumor lesions, were highly inconsistent. Results are depicted in table 2.

Table 2. PCR of skin swab samples

		Skin		Tumor	
Location	*ID*	*primary PCR*	*nested PCR*	*primary PCR*	*nested PCR*
Culebrita –	G1	-	-	N/A	N/A
Green turtles (*Chelonia mydas*)	G2	-	-	N/A	N/A
	G3	-	-	N/A	N/A
	G12	-	+	N/A	N/A
	G13	-	-	N/A	N/A
	G14	-	+	N/A	N/A
	G15	-	+	N/A	N/A
Puerto Manglar –	G4	-	-	N/A	N/A
Green turtles	G5	-	-	-	-
(*Chelonia mydas*)	G6	-	+	N/A	N/A
	G7	-	-	-	-
	G8	-	-	-	-
	G9	-	-	N/A	N/A
	G10	-	-	-	-
	G11	-	+	N/A	N/A
	G16	-	-	+	+
	G17	-	-	-	+
Carlos Rosario –	H1	-	+	N/A	N/A
Hawksbills	H2	-	+	N/A	N/A
(*Eretmochelys imbricata*)	H3	-	+	N/A	N/A
	H4	-	+	N/A	N/A
	H5	-	+	N/A	N/A
	H6	-	+	N/A	N/A
	H7	-	+	N/A	N/A

Histopathology

Green sea turtles:

In general, the skin in grossly normal areas consisted of a 5 to 20 cell-thick epidermis with variable levels of keratinization. In the dermis, blood vessels were arranged in small clusters of vascular channels surrounded by connective tissue and scattered melanocytes. This perivascular connective tissue was often infiltrated, in variable degrees, by lymphocytes and plasma cells.

Out of seven individuals with gross lesions consistent with FP, six had histopathologic findings suggestive of the infection. These lesions consisted of well demarcated, non-encapsulated fibroblast proliferations in the dermis. In a few cases there were concomitant epidermal papillary projections.

Interestingly enough, blood fluke eggs were observed lodged in dermal capillaries of three animals. These lodged eggs were surrounded by a marked heterophilic granulomatous reaction. One of these animals had no gross lesions consistent with FP and this finding was interpreted to be incidental. The other two individuals had in fact gross lesions consistent with FP. However, one of these "grossly positive" animals had no histologic or molecular (PCR) evidence of FP infection, because the only gross lesion observed on this animal was located in close proximity to the eye, and no tumor biopsy was taken. Histopathologic findings of the gross papillomatous lesion consisted of a heterophilic granuloma, most likely in reaction to a lodged fluke egg. The remaining animal was positive for the presence of GTHV by PCR.

Hawksbills:

The skin of hawksbills, although very similar to the green sea turtles, in the few (7) cases we examined, showed subtle differences. The epidermis appeared to be slightly thinner and dermal blood vessels were seen individually and in clusters. In those arranged in clusters, there was rare-to-minimal lympho-plasmacytic infiltration and moderate numbers of melanocytes. In the dermis of two individuals blood fluke eggs were observed lodged in capillaries. However, the surrounding inflammatory reaction appeared milder than in green sea turtles.

Discussion

Herpesviral DNA was detected in 4 out of 6 of the tumor biopsies, demonstrating a herpesvirus association with fibropapillomas in the green turtles of Puerto Manglar. One of these cases (G17) was not, in fact, a fibropapilloma but rather was shown by histopathology to be a granulomatous reaction to the presence of lodged eggs of blood flukes. The other case (G10), had histologic characteristics of a regressing tumor, in that histopathology showed the base of the tumor was infiltrated by numerous lymphocytes, plasma cells, and macrophages. In a previous study, using real time quantitative PCR, variation in viral load was found among tumor samples and it was hypothesized that this variation was related to tumor stage[10].

All grossly normal skin from both affected and unaffected animals was negative by PCR and most tumors were positive, which indicates that virus is probably localized to the focal areas in the skin where it causes tumors, rather than being widely disseminated throughout the epidermis. If this is true, attempts to determine the presence of virus through skin sampling prior to the demonstration of gross FP lesions is not a practical approach.

Based on the biopsy and histopathology results of this study, Culebrita and Manglar show obvious differences in FP-prevalence despite their close geographic proximity. This situation is not entirely unique. Similar examples of this occurrence have been reported in Florida and Hawaii[11,12]. There has for some time been suspicion surrounding the involvement of environmental factors in the development of fibropapillomas[13]. The prevalence of FP seems to be associated with certain environmental characteristics, particularly near-shore bays and lagoons bordered by agricultural and urban development[13]. The Puerto Manglar survey site fits this description. Whether or not environmental factors contribute extensively to this particular disease, there is an obvious indication for the need to protect the habitats of these endangered turtles from the destructive effects of human activity.

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[10] Quackenbush SL, Casey RN, Murcek RJ, Paul TA, Work TM, Limpus CJ, Chaves A, duToit L, Perez JV, Aguirre AA, Spraker TR, Horrocks JA, Vermeer LA, Balazs GH, Casey JW. (2001) Quantitative Analysis of herpesvirus Sequences from Normal Tissue and Fibropapillomas of Marine Turtles with Real-Time PCR. Virology 287: 105-111.

[11] Balazs GH. (1991) Current status of fibropapillomas in the Hawaiian green turtle, *Chelonia mydas*. Research Plan for Marine Turtle Fibropapilloma, US Department of Commerce, NOAA Tech Memo NMFS-SWFSC-156.

[12] Herbst LH. (1994) Fibropapillomatosis of marine turtles. Annual Review of Fish Diseases 4: 389-425.

[13] Aguirre AA. (1998) Fibropapillomas in marine turtles: a workshop at the 18th Annual Symposium on Biology and Conservation of Sea Turtles: 10-12.

The skin swabs were not found to be consistent with biopsy PCR or histopathology. Swabs of twelve animals with no gross lesions were found to be positive for GTHV by nested PCR, but nested PCR of tumor swabs failed to detect viral sequences for all but two of the tumors sampled, one of which was determined not to be a fibropapilloma based on biopsy PCR and histopathology. In light of the biopsy results, it seems highly unlikely that viral particles would be present on the surface epithelium of unaffected skin. Many studies have shown an inability to amplify herpesviral sequences from normal non-tumored animals[14,15,11]. However, in one study in which herpesviral sequences were detected in skin samples from five non-tumored animals, the viral loads in these tissues were much lower than in turtles with fibropapillomas, and that the number of viral DNA copies per cell was significantly higher in tumor tissue than in unaffected tissues of turtles with FP[12]. Therefore, if viral DNA could be amplified from unaffected skin taken from animals of healthy appearance, it would imply that viral DNA should also be detectable in the tumor itself, which is more likely to contain high levels of viral DNA

Based on the results of this study, the swab technique does not appear to be useful. Almost all of the swab results that showed poor correlation to biopsy and histopathology were exhibited by nested PCR only. This is understandable since nested PCR is extremely sensitive to contamination. The swab technique showed poor sensitivity and specificity as compared to biopsy PCR and histopathology, detecting virus in only one of the FP positive tumors and exhibiting a positive result for nested PCR for the tumor that was shown to be negative for GTHV by biopsy PCR and to be a granuloma by histopathology.

A better understanding of the role of GTHV in the pathogenesis of this disease may ultimately require isolation of the virus and fulfillment of Koch's postulates. Until that is possible, continued monitoring of affected populations may provide useful insight into the mechanisms of this disease both within the infected individual and within the population. It is important to remember that all disease states result from the interaction between host, pathogen, and environment. Even without a clear understanding of this disease, it is obvious that the most important measures we can take to decrease the negative impact of FP on endangered sea turtle populations would be to increase the overall health of our oceans by working to decrease the negative human impacts where possible.

[14] Quackenbush SL, Work TM, Balazs GH, Casey RN, Rovnak J, Chaves A, duToit L, Baines JD, Parrish CR, Bowser PR, Casey JW. (1998) Three closely related herpesviruses are associated with fibropapillomatosis in marine turtles. Virology 246: 392-399.

[15] Lackovich JK, Brown DR, Homer BL, Garber RL, Mader DR, Moretti RH, Patterson AD, Herbst LH, Oros J, Jacobson ER, Curry SS, and Klein PA. (1999) Association of herpesvirus with fibropapillomatosis of the green sea turtle *Chelonia mydas* and the loggerhead turtle *Caretta caretta* in Florida. Diseases of Aquatic Organisms 37(2): 89-97.